Evaluation of acute and sub-chronic toxicity of Semelil (ANGIPARS™), a new phytotherapeutic drug for wound healing in rodents

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ABSTRACT

Semelil (ANGIPARS™), an herbal formulation containing *Melilotus officinalis* extract, is a novel compound being developed for treatment of chronic wounds, particularly diabetic foot ulcers. The purpose of this study was to investigate toxicological, pharmacological, and pathomorphological effects of I.M. and I.P. administration of Semelil in animals.

The acute toxicity parameters of Semelil diluted in normal saline (1:10 or 1:5) were determined after a single injection into BALB/c mice and Wistar rats in two steps. First, the LD_{50} was approximately assessed and then the precise lethal dose indices were estimated by the probit-analysis method. Specific single-dose effects of Semelil were monitored for clinical signs of toxicity, including general state of the animals, changes in their behavior, hematological and biochemical parameters for 14 days after drug administration. Then, subacute-chronic toxicity was evaluated in rats treated with Semelil for 3 months.

In acute toxicity study, the calculated LD_{50} for drug diluted at 1:5 was in the range of 44-52 ml/kg. The adverse effects at drug doses close to the LD_{50} included depressed mood, narcosis, and sleep. No adverse pharmacological or toxicological effects of the drug diluted at 1:10 and administered in the single-dose (25-50 ml/kg body wt.) or chronically (daily doses of 0.07 and 0.21 ml/kg body wt.) were noted. Thus, the animal studies demonstrated a favorable safety profile for the phytotherapeutic Semelil.

Keywords: Semelil, ANGIPARS™, Preclinical trials, Rodents, Toxicity, LD_{50}

INTRODUCTION

Among patients with diabetes mellitus, up to 15% will experience a foot ulcer in their lifetime (1), and that is a major risk factor for future lower-extremity amputation in diabetics (2-4). In spite of all therapeutic efforts to prevent amputation in diabetic patients in the last decade, the incidence of lower-extremity amputation is rising both in developed and developing countries (5,6). Therefore, new appropriate drugs to heal diabetic wound are essential. There are several recognized methods for treatment of sub-acute diabetic wounds. However, there is a need for alternative and complementary pharmacotherapy that provides accelerated healing and decrease the rate of recurrent ulcer complications and prevent major surgery and limb amputation. Herbal extracts are among medications with wound healing properties that can be applied more easily on wounds. Previous studies have reported a potential usefulness of internal and external administration of *Melilotus officinalis* (Yellow Sweet Clover) extract for micro-circulation improvement in leg chronic venous insufficiency and hydrocortisone-like anti-inflammatory effects (7,8). The activity of extract of Herba Meliloti in combination *with Dipteryx odorata* and *Ginkgo biloba* was very significant for treatment of lymphedema of lower limbs (7-9). According to these known effects of *Melilotus officinalis*, a novel herbal-based compound was supposed to cure chronic wounds, particularly diabetic foot ulcers and was formulated. Advantageously, this drug "Semelil" have provided full healing with accelerated wound closure. Surprisingly, it also improved the quality of tissue in the healing

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wound with very noticeable hair growth on the scar tissue that ensured a lower rate of ulcer recurrence in the future.

The aim of this study was to evaluate safety of Semelil as a new candidate drug for wound healing in toxicity tests in rodent.

MATERIALS AND METHODS

Semelil herbal extract (ANGIPARS™) was prepared and delivered by ParsRoos Co. (Tehran, Iran). Total protein, creatinine, urea, total bilirubin, of lactate dehydrogenase and alkaline phosphatase activity were determined by a set of reagents of Diakom-Sinteco (Russia). The triglyceride and cholesterol levels were measured by a set of reagents of DiaSys Diagnostic Systems GmbH. (Germany). The activity of alanine aminoteransferase was determined using a set of reagents of Corway (Poland). All other biochemical parameters and enzyme activities were determined using a biochemical semiautomatic analyzer FP-901 (Labsystems, Finland). The blood elements were counted using automatic cell counter Picoskel (Hungary). The glucose level was measured by a set of reagents of the Labsystems Co. (Helsinki, Finland)

Drug solution preparation

ANGIPARS™ was freshly diluted in sterile saline (0.9% sodium chloride) at 1:5 or 1:10 ratios and then different portions of the solution were administered to animals.

Animals

BALB/c mice and Wistar rats were employed for administration of the preparation intramuscularly (in mice) and intraperitoneally (in mice and rats). The protocol of the study was approved by the Center Animals Ethics Committee.

The animals, male and female BALB/c mice (18-20g) and Wistar rats (120-180g) that had a veterinary and health certificates were taken from Laboratory animal farm Stolbovaya (RAMS, Russia) and the animals were kept in type T-3 (mice) and T-4 (rats) cages (Velaz, Prague, Czech Republic), 7-8 animals in each, at 20-22ºC and 60-65% humidity under a 12h light/dark cycle and had free access to food and tap water.

Single-dose toxicity evaluation

The toxicity of diluted Semelil was determined after a single injection into animals (male and female of 128 mice and 48 rats) in two steps. First, the LD_{50} of Semelil intramuscularly (in mice) and intraperitoneally (in mice and rats) were approximately established with acute dose schedule used as recommended by Deichmann and LeBlanc (10). The precise fatal toxicity indices of LD_{16}, LD_{50} and LD_{84} were determined by the probit-analysis method of Litchfield and Wilcoxon (12). Then, single sub-acute doses of the drug were injected by I.M. and I.P. routs and the animals were observed for viability and clinical signs of toxicity on the day of dosing and then daily for 14 days (11).

Sub-chronic toxicity evaluation

Preliminary chronic safety studies with Semelil were performed on 90 male and female Wistar rats at sub-acute doses for 3 months. The rats were divided into 3 groups, 30 animals in each (15 male and 15 female) and Semelil was administered at doses of 0.07 ml/kg and 0.21 ml/kg once a day intramuscularly to the 2nd and 3rd groups, respectively. The first group received sterile solution of sodium chloride 0.9% as control. During the experiment, general state including the dynamics of body weight changes, appetite, motor activity, hair condition and behavior of the animals were recorded (13,14).

Blood samples (2.0-2.5 ml) were taken from caudal vein of each animal for hematological and biochemical analyses at the start of the study and 1 and 3 months after the first administration of drug or saline.

At the end of the chronic experiment, the animals' euthanasia was performed by diethyl ether narcosis and pathomorphology of the internal organs and tissues of the animals were examined. The autopsy of animals was performed immediately after death according to the pathoanatomic scheme, which reduced the possibility of autolysis.

Statistical Analyses

All measured variables were demonstrated as mean and SD. Statistical analyses were performed by using SPSS software, version 11.5 and Stata version 8. The probability levels of significance were based on Student's t-test or non-parametric test, considering the normality of tested variable. Statistically significance level was set at $p<0.05$.

RESULTS AND DISCUSSION

Single dose toxicity evaluation

The results of acute intoxication study after single-dose of the drug introduced to BALB/c mice and Wistar rats and calculated I.M./I.P. fatal toxicity indices are presented in Table 1. The single i.m. injection of Semelil diluted in sterile saline at 1: 10 ratio at the doses of 0.5-1 ml/mouse (25-50 ml/kg) did not cause any toxicity and death. Increasing the dose to 1.5-2 ml/mouse (75-100 ml/kg) remarkably decreased motor activity in mice and caused some deaths. The

Table 1. Acute toxicity parameters of Semelil and ethanol 96% diluted (1:5) in solution of sodium chloride 0.9% following I.M. or I.P. administration to mice and rats.

Fatal toxicity indices, ml/kg						
	Males			Females		
Routes of adminstration	LD_{16}	$LD_{50\pm}SD$	LD_{84}	LD_{16}	$LD_{50\pm}SD$	LD_{84}
BALB/c mice, Semelil						
Intramuscular	35.5	51.8±8.6	81.6	29.2	48.5±4.6	78.3
Intraperitoneal	28.4	47.7±4.9	77.5	25.6	44.9±5.3	74.7
BALB/c mice, Ethanol 96%						
Intraperitoneal	30.4	37.3±2.1	44.8	28.3	35.2	42.7
Wistar rats, Semelil						
Intraperitoneal	25.3	44.6±6.3	74.4	22.7	42.1±5.7	71.8

The data are presented as mean ± standard deviation (SD); LD= lethal dose.

Table 2. Hematological parameters in male rats (3-month study) after Semelil I.M. administration at the dose of 0.07 and 0.21 ml/kg once a day. The control group received 0.9% sodium chloride.

Observation period	Control	Semelil 0.07 ml/kg	Semelil 0.21 ml/kg
Erythrocytes, 10^{12}/l			
Before introduction	7.5 ± 1.2	7.7 ± 1.2	7.4 ± 1.2
1 month after introduction	7.4 ± 1.6	7.3 ± 1.6	7.5 ± 1.6
3 months after introduction	7.6 ± 1.2	7.5 ± 1.2	7.4 ± 1.2
Leukocytes, 10^{9}/l			
Before introduction	11.7 ± 2	12.1 ± 1.6	11.9 ± 1.6
1 month after introduction	12.03 ± 2.8	12.6 ± 1.2	12.1 ± 1.2
3 months after introduction	12.1 ± 2	12.3 ± 1.6	12.2 ± 2
Thrombocytes, 10^{9}/l			
Before introduction	692 ± 116	705 ± 88	685 ± 124
1 month after introduction	701 ± 124	693 ± 96	699 ± 104
3 months after introduction	697 ± 92	702 ± 124	707± 128
Hemoglobin, g/l			
Before introduction	113 ± 12	111 ± 16	114 ± 12
1 month after introduction	111 ± 12	116 ± 16	115 ± 20
3 months after introduction	115± 16	114 ± 12	116 ± 16

Data are means ± standard deviation; All significance levels between groups were p>0.05.

intramuscular injection of Semelil diluted at 1:5 ratios was painful and caused profound depression and some deaths. In acute toxicity study, the calculated LD_{50} for drug diluted at 1:5 was in the range of 44-52 ml/kg. There was no significant difference ($p > 0.05$) between acute toxicity data obtained in experiments using I.M. and I.P. routes of drug administration (Table 1).

I.M. and I.P. injection of Semelil to BALB/c mice, in doses close to LD_{50}, was accompanied by clear depression, narcosis and sleep in animals. In the autopsy of animals plethora of the internal organs was observed. The clinical picture of the intoxication of Wistar rats with equivalent doses of Semelil at 1:5 dilution was similar to that of mice.

No specific and sex-related differences in the sensitivity of the animal species to the toxic effects of Semelil were detected when it was administered to BALB/c mice and Wistar rats intraperitoneally.

Table 3. Hematological parameters in female rats (3-month study) after Semelil I.M. administration at the dose of 0.07 and 0.21 ml/kg once a day. The control group received 0.9% sodium chloride.

Observation period	Control	Semelil 0.07 ml/kg	Semelil 0.21 ml/kg
Erythrocytes, 10^{12}/l			
Before introduction	7.4 ± 1.2	7.5 ± 1.6	7.4 ± 1.2
1 month after introduction	7.5 ± 1.6	7.7 ± 1.2	7.3 ± 1.6
3 months after introduction	7.6 ± 1.2	7.3 ± 1.2	7.5 ± 1.2
Leukocytes, 10^{9}/l			
Before introduction	11.8 ± 1.6	12.5 ± 2.4	11.9 ± 1.6
1 month after introduction	12.0 ± 1.2	12.3 ± 1.2	12.3 ± 1.2
3 months after introduction	12.1 ± 0.8	11.9 ± 3.6	11.6 ± 1.2
Thrombocytes, 10^{9}/l			
Before introduction	703 ± 116	692 ± 116	701 ± 92
1 month after introduction	708 ± 124	699 ± 104	705 ± 104
3 months after introduction	697 ± 104	705 ± 124	697 ± 116
Hemoglobin, g/l			
Before introduction	113 ± 16	114 ± 12	116 ± 16
1 month after introduction	112 ± 32	116 ± 12	118 ± 12
3 months after introduction	115 ± 16	118 ± 16	117 ± 12

Data are means ± standard deviation; All significance levels between groups were $p>0.05$.

Table 4. Hepatic enzyme activity of male rats (3-month study) after Semelil I.M. administration at the dose of 0.07 and 0.21 ml/kg once a day. The control group received 0.9% sodium chloride.

Observation period	Control	Semelil 0.07 ml/kg	Semelil 0.21 ml/kg
Alkaline phosphatase, unit/l			
Before introduction	504.9 ± 177	499.4 ± 154	516.8 ± 128
1 month after introduction	480.8 ± 108	486.2 ± 175	514.9 ± 131
3 months after introduction	469.7 ± 159	487.7 ± 145	494.4 ± 136
Alanine aminotransferase, unit/l			
Before introduction	65.82 ± 23	60.81 ± 22	61.84 ± 16
1 month after introduction	67.21 ± 25	64.91 ± 18	66.29 ± 17
3 months after introduction	60.85 ± 18	62.69 ± 20	62.71 ± 13
Aspartate aminotransferase, unit/l			
Before introduction	90.87 ± 33	91.38 ± 36	89.32 ± 26
1 month after introduction	81.11 ± 32	84.44 ± 28	85.73 ± 36
3 months after introduction	87.92 ± 26	86.66 ± 26	84.31 ± 35
Lactate dehydrogenase, unit/l			
Before introduction	747.38 ± 240	809.84 ± 165	798.36 ± 209
1 month after introduction	820.26 ± 275	772.73 ± 190	771.44 ± 154
3 months after introduction	738.63 ± 259	794.77 ± 246	797.36 ± 138

Data are means ± standard deviation; All significance levels between groups were $p>0.05$.

Table 5. Hepatic enzyme activity of female rats (3-month study) after Semelil I.M. administration at the dose of 0.07 and 0.21 ml/kg once a day . The control group received 0.9% sodium chloride.

Observation period	Control	Semelil 0.07 ml/kg	Semelil 0.21 ml/kg
Alkaline phosphatase, unit/l			
Before introduction	494.9 ± 151	501.4 ± 184	508.7 ± 192
1 month after introduction	509.2 ± 154	558.1 ± 154	489.4 ± 156
3 month after introduction	500.3 ± 196	509.8 ± 153	491.9 ± 156
Alanine aminotransferase, unit/l			
Before introduction	62.84 ± 21	68.82 ± 19	62.08 ± 23
1 month after introduction	59.37 ± 21	64.29 ± 23	68.78 ± 17
3 months after introduction	61.86 ± 25	62.04 ± 21	59.89 ± 21
Aspartate aminotransferase, unit/l			
Before introduction	92.61 ± 39	100.88 ± 34	99.46 ± 30
1 month after introduction	96.24 ± 25	102.76 ± 33	97.82 ± 33
3 months after introduction	92.88 ± 37	108.27 ± 33	94.29 ± 36
Lactate dehydrogenase, unit/l			
Before introduction	857.28 ± 193	879.84 ± 205	902.35 ± 209
1 month after introduction	801.30 ± 166	887.64 ± 186	863.76 ± 239
1 months after introduction	798.32 ± 169	861.82 ± 153	875.24 ± 207

Data are means ± standard deviation; All significance levels between groups were p>0.05.

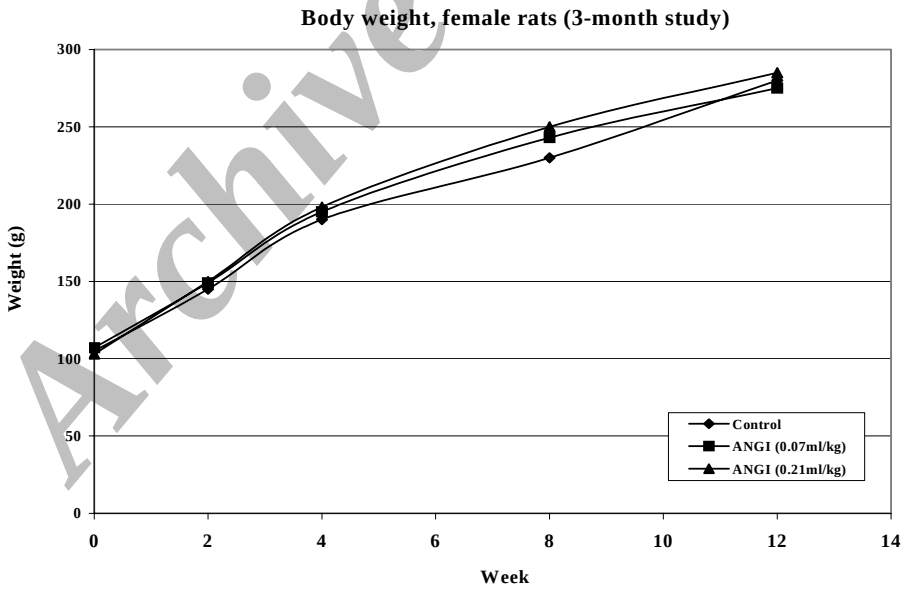

Figure 1. The dynamics of body weight changes in female rats (3-month study) following Semelil I.M. administration at the dose of 0.07 and 0.21 ml/kg once a day. The control group received 0.9% sodium chloride.

Table 6.Biochemical parameters in male rats (3-month study) after Semelil I.M. administration at the dose of 0.07 and 0.21 ml/kg once a day . The control group received 0.9% sodium chloride.

Observation period	Control	Semelil 0.07 ml/kg	Semelil 0.21 ml/kg
	Total protein, g/l		
Before introduction	61.8 ± 11.5	60.2 ± 14	62.8 ± 8
1 month after introduction	63.7 ± 9.5	66.0 ± 10	66.7 ± 6.9
3 months after introduction	61.9 ± 13.5	62.5 ± 13.7	64.4 ± 10.8
	Bilirubin, µmol/l		
Before introduction	7.29 ± 2	7.89 ± 2.5	7.69 ± 1.5
1 month after introduction	7.39 ± 1.8	7.80 ± 2	7.85 ± 2
3 months after introduction	7.58 ± 1.5	7.78 ± 1.5	7.69 ± 1.8
	Urea, mmol/l		
Before introduction	10.48 ± 2.5	11.81 ± 3.5	10.28 ± 3.5
1 month after introduction	11.16 ± 1.7	10.47 ± 2.2	10.36 ± 2.5
3 months after introduction	10.35 ± 2.6	10.72 ± 2.1	11.27 ± 2.6
	Creatinine, µmol/l		
Before introduction	58.82± 22	61.75 ± 21	62.13 ± 21
1 month after introduction	64.66 ± 26	66.12 ± 22.6	63.52 ± 16.6
3 months after introduction	63.02 ± 21	60.09 ± 16.6	69.69 ± 20.7
	Glucose, µmol/l		
Before introduction	7.43 ± 1.3	7.58 ± 1.4	7.34 ± 1.0
1 month after introduction	7.44 ± 1.1	7.33 ± 1.1	7.52 ± 1.2
3 months after introduction	7.45 ± 0.8	7.47 ± 1.2	7.44 ± 0.9
	Total cholesterol, mmol/l		
Before introduction	1.16 ± 0.32	1.14 ± 0.36	1.28 ± 0.76
1 month after introduction	1.18 ± 0.28	1.04 ± 0.32	1.36 ± 0.56
3 months after introduction	1.32 ± 0.36	1.16 ± 0.4	1.27 ± 0.32
	Triglycerides, mmol/l		
Before introduction	0.67 ± 0.2	0.71 ± 0.32	0.70 ± 0.28
1 month after introduction	0.62 ± 0.2	0.75 ± 0.48	0.67 ± 0.32
3 months after introduction	0.55 ± 0.32	0.69 ± 0.24	0.79 ± 0.44

Data are means ± standard deviation; All significance levels between groups were p>0.05.

Figure 2. The dynamics of body weight changes in male rats (3-month study) following Semelil I.M. administration at the dose of 0.07 and 0.21 ml/kg once a day. The control group received 0.9% sodium chloride.

Table 7. Biochemical parameters in female rats (3-month study) after Semelil I.M. administration at the dose of 0.07 and 0.21 ml/kg once a day . The control group received 0.9% sodium chloride.

Observation period	Control	Semelil 0.07 ml/kg	Semelil 0.21 ml/kg
	Total protein, g/l		
Before introduction	62.7 ± 9.5	62.6 ± 10.7	66.4 ± 9.4
1 month after introduction	63.1 ± 10.3	63.09 ± 11.3	66.8 ± 9.5
3 months after introduction	62.9± 9.7	62.6 ± 9.6	64.5 ± 8.9
	Bilirubin, µmol/l		
Before introduction	7.61 ± 2.2	7.88 ± 1.9	7.59 ± 1.8
1 month after introduction	7.28 ± 1.9	7.62 ± 1.9	7.48 ± 1.7
3 months after introduction	7.86 ± 2	7.94 ± 2.1	7.69 ± 1.9
	Urea, mmol/l		
Before introduction	10.82 ± 2.1	11.06 ± 2.7	10.87 ± 3
1 month after introduction	10.38 ± 2.3	10.28 ± 2.5	10.29 ± 2.3
3 months after introduction	10.28 ± 2	10.36 ± 2.2	10.20 ± 2
	Creatinine, µmol/l		
Before introduction	59.62 ± 18.5	64.85 ± 23.2	62.18 ± 17.9
1 month after introduction	62.58 ± 17.3	48.78 ± 19.4	65.23 ± 17.3
3 months after introduction	60.29 ± 17.4	66.82 ± 18.5	61.41 ± 20.1
	Glucose, µmol/l		
Before introduction	7.38 ± 1.2	7.39 ± 1.3	7.59 ± 1.4
1 month after introduction	7.36 ± 1.1	7.58 ± 1.5	7.48 ± 1.2
3 months after introduction	7.39 ± 1.5	7.49 ± 1.0	7.69 ± 1.2
	Total cholesterol, mmol/l		
Before introduction	1.19 ± 0.32	1.09 ± 0.36	1.28 ± 0.32
1 month after introduction	1.29 ± 0.32	1.28 ± 0.32	1.28 ± 0.36
3 months after introduction	1.26 ± 0.36	1.19 ± 0.32	1.26 ± 0.36
	Triglycerides, mmol/l		
Before introduction	0.70 ± 0.28	0.69 ± 0.28	0.71 ± 0.32
1 month after introduction	0.78 ± 0.32	0.72 ± 0.36	0.78 ± 0.36
3 months after introduction	0.72 ± 0.36	0.76 ± 0.36	0.79 ± 0.36

Data are means ± standard deviation; All significance levels between groups were p>0.05.

Sub-chronic toxicity evaluation

Daily I.M. injections of Semelil at the doses of 0.07 and 0.21 ml/kg body weight had no effect on general state and behavior of rats. We did not detect any significant changes in hematological and biochemical parameters including total serum proteins, bilirubin, glucose content, triglycerides, cholesterol, and blood urea and creatinine, in test animal groups compared to control group after 1 and 3 months in rats. The activities of hepatic enzymes of test and control groups did not show any difference and corresponded to their physiological ranges, which are typical for these animals (Tables 1-7).

Mean body weights of rats in experimental groups, whether they received the preparation in doses of 0.07 or 0.21 ml/kg during all chronic toxicity experiment, were not significantly different ($p > 0.05$) from those of control animals (Fig. 1,2).

Pathomorphological examination of animals after chronic experiment

At necropsy, macroscopic evaluation of the animals together with microscopic data showed that both test and control groups were practically healthy. No toxic or toxico-allergic effects of Semelil were revealed in the test groups (2^{nd} and 3^{rd}) of rats. No pathological changes of the internal organs and no local-irritating effects of the preparation were discovered in both test groups during the study period Our experiments showed that Semelil is a low-toxic substance after I.M. and I.P. administration to BALB/c mice and

Wistar rats. Since there were no drug adverse effects and the calculated LD_{50} was in the range of 44-52 ml/kg in the acute dose study, it can be concluded that Semelil was well tolerated in rats both at I.M. administered doses of 0.07 and 0.21 ml/kg, which were used in the sub-acute chronic toxicity study.

Considering the profound effects of intraperitoneal and intramuscular injections of Semelil at dose levels close to the LD_{50} such as depression, narcosis and sleep, and their similarity to intoxication by ethanol (Table 1), which was used for the preparation of the drug, it can be assumed that these effects were directly related to this solvent.

The data indicated the stability of protein-productive function of liver, hepatic enzyme activity, carbohydrate metabolism, pancreas function, lipid metabolism, excretory kidney function of animals during the study period because we did not observe significant changes in recorded hematological and biochemical parameters in test groups compared to control group.

CONCLUSION

Results of the present study gave an evidence of good tolerance of Semelil and the absence of undesirable effects on the functional state of the vital organs of the experimental animals in sub-acute and chronic test. Since antioxidants are believed to be useful in the management of diabetes and its complications (15) and regarding antioxidant potential of Semelil the next step of trials can be focused on diabetes.

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